Dear participants in the *P. ramorum* Provisional Approval Program,

A new proficiency panel for the *P. ramorum* Provisional Approval (PA) program should be ready for distribution in two weeks.

The PA program for *P. ramorum* is continuing to evolve and move forward. We now have a full year of inspections, training and proficiency testing completed and much has been learned and improved. The Real-Time PCR assay for *P. ramorum* diagnostics was validated and approved for use in 2005. Participating laboratories now have a choice of two approved molecular tests, conventional (nested) PCR and Real-Time PCR, for both routine diagnostics and for use in proficiency panel evaluation. This year, panels will be provided from April through June and each lab will identify which month they prefer to receive a panel(s).

There are now over 20 labs participating in the PA program. There are three categories of labs: (1) labs and personnel that were already provisionally approved in 2005 for the conventional PCR diagnostic and now must renew their PA for 2006 by passing a maintenance panel. If these labs also wish to perform Real-Time PCR diagnostics and had suitable equipment on hand during their initial 2005 lab inspection, the maintenance panel can be used to both renew the PA for conventional PCR and to add PA for Real-Time PCR. If no Real-Time PCR equipment was purchased and/or there is no interest in adding Real-Time PCR, then these labs will simply use their maintenance panel for conventional PCR PA; (2) Labs that have previously requested, but have not yet received panels, need to request their initial panel(s) for conventional PCR and indicate if they wish to also have Real-Time PCR PA. Again, suitable Real-Time PCR equipment must have been on hand during the time of the initial lab inspection; (3) Labs that have been inspected but have not previously requested panels should indicate their status so that we can be prepared to deliver the initial panel(s) at the requested time.

If you wish to have PA for *P. ramorum* diagnostics using Real-Time PCR, we are requiring PA in conventional PCR prior to or concurrent with approval for performing the Real-Time PCR. You may choose to use the Real-Time diagnostic in your routine work but you must be proficient in the conventional assay also. Current CPHST work instructions require the use of both diagnostic methods when certain Real-Time or conventional PCR results are obtained. Out-of-range data obtained by Real-Time PCR can usually be resolved by running the nested PCR protocol on the suspect samples. Being proficient in both diagnostics allows you the ability to crosscheck your assays. Another advantage of maintaining proficiency in both diagnostic protocols is that you can continue processing samples if an instrument should require repair.

We would appreciate your completing the table on page 4 and returning it to us by e-mail. Please make sure the following information is included:

1. List each diagnostician who will routinely perform the testing and indicate whether he/she has been previously provisionally approved. A panel for each diagnostician who will perform the routine testing is required, irrespective of whether the panel is for a new PA or for maintenance of PA.
2. Panels are in limited supply, so ask only for panels your lab requires and can process in a timely manner.
3. Select a time that fits with your lab schedule:

April	Receipt week one	Return first week of May
May	Receipt week one	Return first week of June
June	Receipt week one	Return first week of July

4. Samples should be analyzed and the results returned no later than 30 days from the scheduled shipment date.
5. Panel samples should be handled (managed, analyzed and reported) in the same manner as real environmental samples utilizing the same trained staff, methods, procedures, equipment and facilities.

6. Proficiency test (PT) samples, or portions of PT samples, are not to be forwarded to another laboratory for analysis.
7. Information on proficiency test samples should not be shared with another laboratory, and must be kept confidential.
8. If training is requested then schedule your panel receipt after the training is concluded.

Depending on when you receive this e-mail, you may have 1 or 2 attachments that include a protocol for conventional (nested and multiplex) PCR, and a protocol for Real-Time PCR. We will be providing additional protocols as each is finalized and approved. We suggest you order primers/ probes/reagents (see tables below) in advance for the method(s) you intend to use so that all required reagents are on hand before you receive your panel(s). Please be aware that fluorescent probes can take as long as 14 days to receive. For those who were already approved we strongly suggest reordering a new set of primers (and probes) since degradation is one of the major reasons for failure or low efficiency of PCR. Please note that the reagents for the conventional assays are the same as those used in the Real-time assay (see table below).

We will make every effort to quickly evaluate results and provide prompt feedback. Unlike the proficiency test of 2005, we will provide an example of the format for you to return your proficiency test results that will include an Excel spreadsheet, and an example of the type of properly labeled gel images (for conventional PCR) that you should return. Returning your panel results in these formats will greatly enhance our ability to expedite the review and approval process. Proficiency panel results that are submitted for review in an unacceptable format will not be reviewed but will be returned for reformatting and resubmission.

Primers used in Conventional PCR for *P. ramorum* diagnostics

Nested Primers:

Primer Name	**Primer Sequence** (synthesized by Integrated DNA Technologies, Inc., Purification - Standard Desalting)
Phyto1 primer	5'-CATGGCGAGCGCTTGA-3'
Phyto2 primer	5'-AAAGCCAAGCCCTGCAC-3'
Phyto3 primer	5'-GGTGGATGGGGACGTG-3'
Phyto4 primer	5'-GAAGCCGCCAACACAAG-3'

Multiplex PCR

Primer Name	**Primer Sequence** (synthesized by Integrated DNA Technologies, Inc., Purification - Standard Desalting)
Platf primer	5'-TTAGTTGGGGGCTTCTGTTC-3'
Platr primer	5'-AGCTGCCAACACAAATTTC-3'
NS1 primer	5'-GTAGTCATATGCTTGTCTC-3'
NS2 primer	5'-GGCTGCTGGCACCAGACTTGC-3'

Primers and Probes used in Real-Time PCR assay for *P. ramorum* diagnostics

Primer Name	**Primer Sequence** (synthesized by Integrated DNA Technologies, Inc., Purification - Standard Desalting)
Pram-114F	5' – TCA TGG CGA GCG CTT GA -3'
Pram-1527-190R	5' – AGT ATA TTC AGT ATT TAG GAA TGG GTT TAA AAA GT – 3'
COX-F	5' – CGT CGC ATT CCA GAT TAT CCA – 3'
COX-RW	5' – CAA CTA CGG ATA TAT AAG RRC CRR AAC TG – 3'
Probe Name	**Probe Sequence** (synthesized by Integrated DNA Technologies, Inc.)
	(Purification - Dual HPLC purification)
Pram1527-134T	5' - /56-FAM/TTC GGG TCT GAG CTA GTA G/3BHQ_1/ - 3'
COX-P	5'- /5TexRd-XN/AGG GCA TTC CAT CCA GCG TAA GCA /3BHQ_2/ - 3'

Reagents used in both the conventional PCR assays and Real-time PCR assays

Platinum *Taq* DNA polymerase Note: Platinum *Taq* is supplied in a kit with 10X PCR Buffer and 50mM $MgCl_2$	10966-034, Invitrogen
dNTP MIX (10 mM solution, PCR reagent)	D-7295, Sigma
D-(+) –Trehalose dehydrate	T9531-10G, Sigma

If you have any questions concerning the provisional approval process in general please contact Dr. Patrick Shiel at (919) 855-7416 or Patrick.J.Shiel@aphis.usda.gov.
Questions concerning the proficiency-testing program should be directed to Dr. Vessela Mavrodieva at (301) 504-7100 ext. 233 (vessela.a.mavrodieva@aphis.usda.gov).

P. ramorum Provisional Approval Program

Lab name:
Fed-Ex shipping address, including phone number:

Diagnostician name	Provisionally approved (PA) for conventional PCR in **2005** **Yes or No**	Seeking Renewal of PA for conventional PCR in 2006 **Yes or No**	Seeking First-time PA for conventional PCR in 2006 **Yes or No**	Seeking to add PA for **Real-Time PCR** in 2006 **Yes or No**	Month that diagnostician wishes to receive and perform test **(April, May, June)**	Requesting training prior to panel receipt **Yes or No**

Please complete the provided form and e-mail it to Patrick Shiel Patrick.J.Shiel@aphis.usda.gov and copy it to Vessela Mavrodieva vessela.a.mavrodieva@aphis.usda.gov.